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Award Number: W81XWH-12-1-0390

TITLE: Connexins and Cadherin Cross-talk in the Pathogenesis of Prostate Cancer

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CONTRACTING ORGANIZATION: University of Nebraska Medical Center, Omaha, NE 68198-7835

REPORT DATE: September 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2013	Annual	1 September 2012 – 31 August 2013

4. TITLE AND SUBTITLE
Connexins and Cadherin Crosstalk in the Pathogenesis
Of Prostate Cancer

5a. CONTRACT NUMBER
W81XWH-12-1-0390

5b. GRANT NUMBER
W81XWH-12-1-0390

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Nebraska Medical Center
987835 Nebraska Medical Center
Omaha, NE 68198-7835

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Gap junctions are ensembles of cell-cell channels that are formed by proteins called connexins. They permit the passage of small molecules between cells and maintain homeostasis. We have found that connexin32 and connexin43 are assembled into gap junctions in normal prostate but remain intracellular in prostate tumors. Our studies showed that the expression of anti-metastatic E-cadherin facilitated gap junction assembly whereas the expression of pro-invasive N-cadherin disrupted assembly. We hypothesized that gap junction assembly was the downstream target of signaling initiated by cadherins. We had proposed 2 specific aims to test this hypothesis. The proposed studies of Aim 1 were to determine how E-cadherin mediated cell-cell adhesion controlled gap junction assembly in prostate cancer cells whereas of aim 2 were to determine the molecular mechanisms by which E-cadherin and N-cadherin modulate gap junction assembly differentially. We have identified key motifs that regulate the endocytosis of connexin43 and connexin32 by clathrin-mediated pathway. Endocytosis of connexin43 is regulated through phosphorylation of serine 279 and 282 via clathrin-mediated pathway whereas that of connexin32 is regulated by three dileucine-like motifs in its cytoplasmic tail. Expression of mutant connexin32, in which the two dileucine-like motifs are mutated, results in the formation of large gap junctions. Finally, we have identified a new post-translational modification of 120-catenin that affects cadherin-connexin cross talk.

15. SUBJECT TERMS
Connexins, gap junctions, cadherins, cell to cell communication, endocytosis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	12	19b. TELEPHONE NUMBER *(include area code)*

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1. Introduction:

Gap junctions (**GJ**) are conglomerations of cell-cell channels that are formed by a family of 21 distinct proteins, called connexin **(Cx)**s. The Cxs are transmembrane proteins, which are designated according to molecular mass. They are assembled into GJs through many steps (**Figure 1**). Communication through GJs is crucial for maintaining homeostasis [1;2]. Impaired, or loss of, Cx expression has been documented in the pathogenesis of various carcinomas [1;3-5]. Moreover, many studies have shown that over-expression of Cxs in tumor cells attenuates the malignant phenotype *in vivo* and *in vitro*, reverses the changes associated with epithelial to mesenchymal transformation (**EMT**), and induces differentiation [3;4;6]. For example, Cx32 is expressed in the liver, lung, and exocrine glands, and knock out studies have shown that the incidence of carcinogen-induced tumors in these mice is higher [7-9]. Moreover, mutations in several Cx genes have been characterized in inherited diseases associated with aberrant proliferation and differentiation [1;10]. These studies support the notion that Cxs act as tumor suppressors. Despite this the molecular mechanisms by which Cxs are assembled into GJs and how GJs are disassembled are poorly understood.

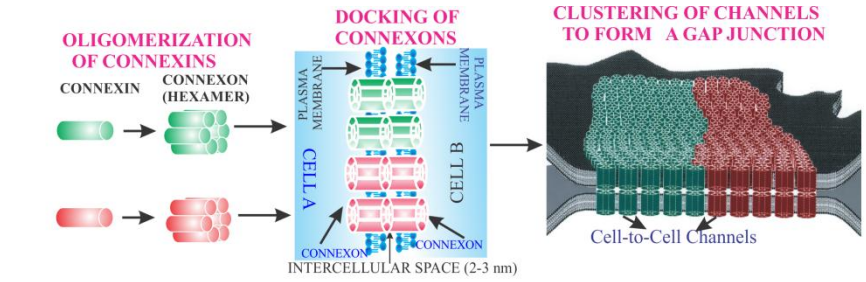

Figure 1. **A cell-cell channel is formed by Cxs, which first oligomerize as hexamers(called connexons or hemichannels), and then are transported to the cell surface and dock with connexons in the adjacent cells to form cell-cell channels. A GJ is formed when several channels cluster at one particular spot. A GJ may be formed of channels formed of more than one Cx. Connexins have 4 transmembrane domains (TM1-TM4 or MI-M4), 2 extracellular loops, and one cytoplasmic domain. The amino and the carboxyl termini face the cytoplasm.**

2. Body

Our central hypothesis is that bidirectional signaling between cadherin(Cad)s and Cxs is required to maintain the polarized and differentiated state of epithelial cells and that GJ assembly is the downstream target of the signaling initiated by the classical Cads, with epithelial (E)-Cad facilitating assembly and neuronal (N)-Cad disrupting the assembly. We had proposed 2 specific aims to test this hypothesis:

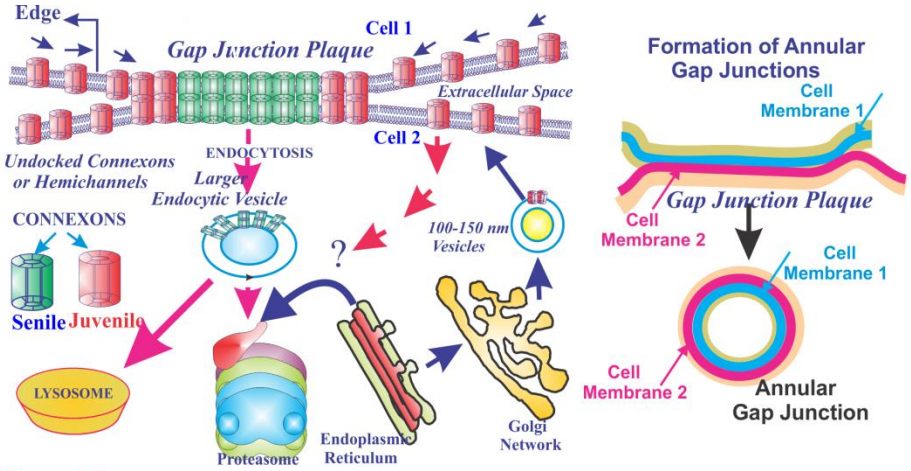

Figure 2. **Assembly and Disassembly of GJs. Cxs are short lived proteins with a half life of 2-5 h. Connexons (see Figure 1) traffic to the plasma membrane (PM) in 100-150 nm particles, diffuse laterally and dock with their counterpart connexons in the PM of apposed cells. Juvenile connexons (red) are recruited to the periphery of the GJ plaque while senile connexons (green) are pinched off from the middle as double membrane vesicles into either one or the other cell. Alternatively, an entire GJ plaque is also endocytosed in its entirety into one or the other cell called annular GJs(left).**

1. **Determine how E-cad mediated cell-cell adhesion controls the assembly of Cxs into gap junctions in human prostate cancer prostate cancer cell lines.**

2. **Determine the molecular mechanisms by which E-Cad and N-Cad modulate gap junction assembly differentially in human prostate cancer cell lines.**

It is as yet unknown how a bi-cellular structure, such as a GJ, also called a GJ plaque, is endocytosed [1;10-12]. Connexins are short-lived proteins and both the assembly of Cxs into GJs and their disassembly are multi-step processes, which are poorly understood (**Figures 1 & 2).** A GJ can be endocytosed into one or the other cell, either in its entirety, also called an annular GJ, or as fragments pinched off from the center of the plaque as double membrane vesicles, by endocytosis and targeted to the lysosome for degradation. Alternatively, undocked connexons may be endocytosed by clathrin mediated or non-clathrin mediated endocytosis (**Figure 2)** [13-16].

Tasks of Aim 1:

1. Prepare recombinant retroviruses that contain various E-cad constructs that alter its ability to mediate cell-cell adhesion.
    a. Prepare recombinant retrovirus containing E-cad (W156A) (**Johnson**).
    b. Prepare recombinant retrovirus containing E-cad with deleted β-catenin binding site (**Johnson**).
    c. Prepare recombinant retrovirus containing E-cad with mutated p120 catenin binding site (**Johnson**).

2. Generate stable polyclonal cultures of several human PC cell lines (LNCaP - **ATCC**; PC3 - **ATCC**; RWPE1 - **ATCC**; PZ-HPV-7 -**ATCC**) expressing the constructs shown in 1 (**Mehta**).

3. In the cells described in 2, determine if connexins are assembled into gap junctions using Triton X-100 solubility assays (**Mehta and Johnson**).

4. In the cells described in 2, determine if cadherins are assembled into adherens junctions using Triton X-100 solubility assays (**Mehta and Johnson**).

5. In the cells described in 2, observe the trafficking of connexins and their assembly into gap junctions (**Mehta and Johnson**).
    a. Perform cell surface biotinylation to detect connexins at the plasma membrane (**Mehta**).
    b. Determine if connexins co-localize with EEA1, clathrin or caveolin-1 (**Mehta and Johnson**).

6. Knock down endogenous E-cadherin in LNCaP prostate cancer cells (**ATCC**) with or without connexin expression (**Mehta**).

    a. Determine if motility is altered in cells expressing E-cadherin or connexins (**Johnson**).

7. Determine if the trafficking of connexins is altered in knock down cells described in 6 (**Mehta**).

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Statement of Work

Aim 1: Determine how E-cadherin mediated cell-cell adhesion controls the assembly of connexins into gap junctions in human prostate cancer cell lines.

Tasks:

1) Prepare recombinant retroviruses that contain various E-cadherin constructs that alter its ability to mediate cell-cell adhesion. (Months 1-9)

- **a) Prepare recombinant retrovirus containing E-cadherinW156A (Johnson).**
- **b) Prepare recombinant retrovirus containing E-cadherinΔβ-cat (Johnson).**
- **c) Prepare recombinant retrovirus containing E-cadherinΔp120 (Johnson).**

Construct E-cadherinW156A in retroviral vector, LZRS, was used to produce recombinant retrovirus. The retroviral construct (20 µg) was transfected in EcoPack cell line and after 48 h the medium containing the virus was collected and filtered (0.45 µM, Millipore, Billerica, MA). To produce recombinant retrovirus for infection of target cells, amphotropic PTi67 cells were infected with the transiently produced recombinant retrovirus from EcoPack and selected in G418 (400 µg/ml). The recombinant retrovirus produced from the pooled polyclonal cultures of PTi67 cells was assayed for the virus titer by colony forming units as described [17-22].

The construct E-cadherinΔβ-cat was prepared as follows: Amino acids 830 through 860 from human E-cadherin, that encompass β-catenin binding site, were deleted (31 amino acid deletion). For engineering the construct E-cadherinΔp120, three amino acid residues (762, 763 and 764) in the cytoplasmic tail of human E-cadherin were mutated to alanines. Thus the sequence was changed from EED to AAA. The construction of these constructs is described [23;24]. We have produced the recombinant retrovirus for E-cadherinΔp120 but not for E-cadherinΔβ-cat.

2) Generate stable polyclonal cultures of several human prostate cancer cell lines (LNCaP - ATCC; PC3 - ATCC; RWPE1 - ATCC; PZ-HPV-7 - ATCC) expressing the constructs shown in 1) (Mehta). (Months 3-12).

We screened several prostate cancer cell lines for the expression E-cadherin and N-cadherin so that we could express the constructs described in task 1. This screen revealed that all the prostate cancer cell lines that we screened expressed E-cadherin or N-cadherin endogenously (**Figure 3**). The characteristics of these cell lines have been described [25-30]. This prohibited us from undertaking the proposed studies in task 2 because the available antibodies for E-cadherin do not distinguish the endogenously expressed E-cadherin from exogenously expressed E-cadherin

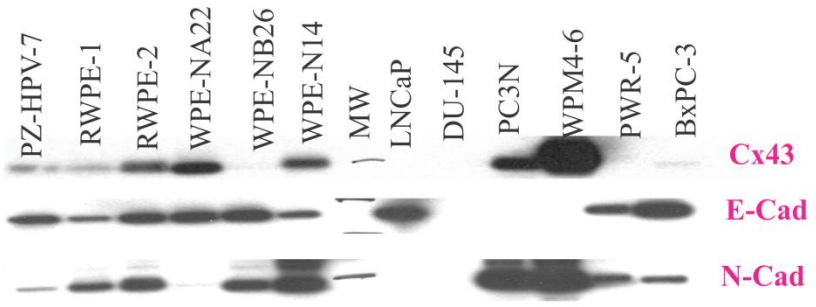

FIGURE 3. We examined the expression of connexins, E-cadherin (E-Cad) and N-cadherin (N-Cad) in all the available, well-characterized prostate cancer cell lines, such as LNCaP, PC3N, and DU-145 as well as in non-transformed cell lines PZ-HPV-7, RWPE-1 and RWPE-2, that have been immortalized by hTERT. All cell lines were from ATCC. WPE-NA22, WPE-NB26, WPE-N14, WPM4-6 and PWR-5 PC cell lines are derived from RPWE-1 after transforming with a chemical carcinogen. BxPC-3 is a pancreatic cancer cell line which expresses E-Cad and N-Cad and was used as a control in these blots. DU-145 cells also express E-Cad, but for reasons not understood, was not detected in this particular blot.

constructs. Introducing these constructs will provide no information with regard to where they are expressed and how they act. These constructs have to be either tagged with Myc or introduced in a prostate cancer cell line that is cadherin null. Alternatively, we will have to design ShRNA for knocking down only the endogenously expressed E-cadherin without knocking down the expression of exogenously introduced engineered E-cadherin constructs. These studies therefore have to be thoughtfully reconsidered and redesigned. One future approach that we would like to undertake is tag both wild type and engineered cadherin constructs with Myc and HA tags, conventionally used by many investigators, to distinguish engineered proteins from endogenously expressed proteins. Highly specific monoclonal and polyclonal antibodies raised against both tags are commercially available and have been used by us in earlier studies [21;23;24;31-34].

The construct E-cadherinW156A, described in task 1a, did not confer cell-cell adhesion when expressed in MiaPaCa cells. These data are significant because they prompt us to undertake similar studies with prostate cancer cell lines that are cadherin null, which we will continue to screen.

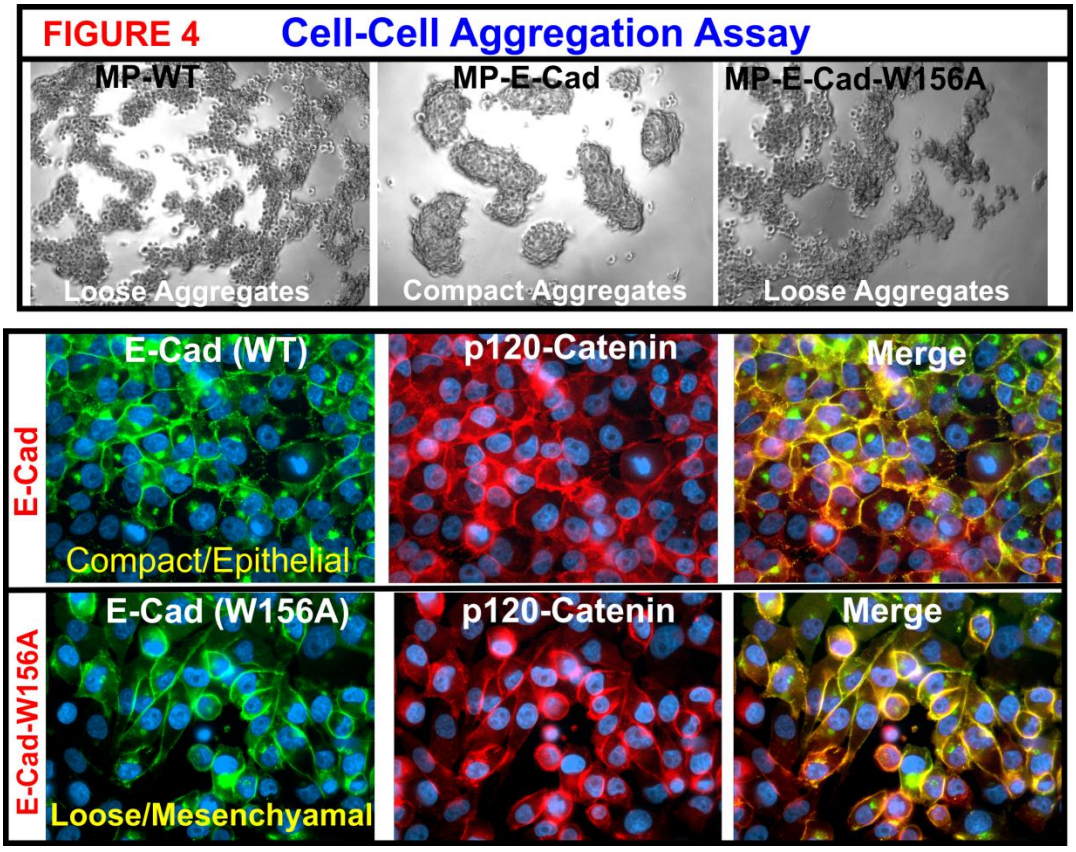

Figure 4. Wild-type (WT) E-cadherin and the mutant E-cadherinW156A (tagged with green fluorescent protein, EGFP) were retrovirally expressed in cadherin-null, human pancreatic cancer cell MiaPaCa. Wild-type and mutant E-cadherin were expressed appropriately at areas of cell-cell contact (**Figure 4, bottom**). As assessed by cell-cell aggregation assays, Cad-null MiaPaCa cells (**MP-WT, Figure 4, top**) and cells expressing mutant Cads (MP-E-Cad-W156A-EGFP) did not adhere and formed loose aggregates, which were dispersed upon trituration (**Figure 4, top**) whereas cells expressing WT E-Cad (**MP-E-Cad, Figure 4, top, middle panels)** formed compact aggregates which could not be dispersed. Both mutant and wild-type Cads were localized at the areas of cell-cell contact **(Figure 4, bottom)**. It is worth noticing that both WT and mutant Cads recruited p120 catenin to cell-cell contact areas (**Figure 4, bottom, middle panel**). In Cad-null MiaPaCa cells, no Cads were seen and p120 catenin was not localized at cell-cell contact areas (data not shown). Cell-cell aggregation assays were performed as described in our earlier studies [18;20].

3) In the cells described in 2), determine if connexins are assembled into gap junctions using Triton X-100 solubility assays (Mehta and Johnson). (Months 4-16)

See comments related to task 2..

4) In the cells described in 2), determine if cadherins are assembled into adherens junctions using Triton X-100 solubility assays (Mehta and Johnson). (Months 4-16)

Please see comments related to task 2.

5) In the cells described in 2), observe the trafficking of connexins and their assembly into gap junctions (Mehta and Johnson). (Months 6-18)
a) Perform cell surface biotinylation to detect connexins at the plasma membrane (Mehta).
b) Determine if connexins co-localize with EEA1, clathrin or caveolin-1 (Mehta and Johnson).

Please see comments related to task 2.

6) Knock down endogenous E-cadherin in LNCaP prostate cancer cells (ATCC) with or without connexin expression (Mehta). (Months 12-20)
a) Determine if motility is altered in cells expressing E-cadherin or connexins (Johnson).

7) Determine if the trafficking of connexins is altered in knock down cells described in 6) (Mehta). (Months 16-24)

We have not initiated these tasks yet.

Aim 2: Determine the molecular mechanisms by which E-cadherin and N-cadherin modulate gap junction assembly differentially in human prostate cancer cell lines.

Tasks:
1) Prepare recombinant retroviruses that contain chimeras of E-cadherin and N-cadherin (Johnson). (Months 6-16)
a) Prepare recombinant retrovirus containing chimeras with the extracellular domains switched (Johnson).
b) Prepare recombinant retrovirus containing chimeras with the cytoplasmic domains switched (Johnson).
c) Prepare recombinant retrovirus containing chimeras with segments of the extracellular domains of E-cadherin and N-cadherin swapped (Johnson).

The preparation of these constructs has been described [31;32]. We have not yet tested these constructs.

2) Infect LNCaP cells (ATCC) and PZ-HPV-7 cells (ATCC) with the retroviruses described in 1) and retroviruses containing wild-type N-cadherin (Mehta). (Months 12-24)

We have not initiated this task. Please see also comments related to task 2 in aim 1.

3) In the cells described in 2), determine if connexins are assembled into gap junctions using Triton X-100 solubility assays (Mehta and Johnson). (Months 16-28)

We have not initiated these studies.

4) In the cells described in 2), determine if cadherins are assembled into adherens junctions using Triton X-100 solubility assays (Mehta and Johnson). (Months 16-28)

We have not initiated these studies.

5) In the cells described in 2), observe the trafficking of connexins and their assembly into gap junctions. (Months 24-32)

a) Perform cell surface biotinylation to detect connexins at the plasma membrane (Mehta).

b) Determine if connexins co-localize with EEA1, clathrin or caveolin-1 (Mehta and Johnson).

We have not initiated this task.

6) Determine if N-cadherin alters the motility of connexin-expressing LNCaP (ATCC) and cells PZ-HPV-7 (ATCC) cells (Johnson). (Months 28-36)

This task has not been initiated yet.

7) Determine if N-cadherin induces endocytosis of gap junctions in connexin-expressing LNCaP (ATCC) and PZ-HPV-7 (ATCC) cells (Mehta). (Months 28-36)

We have not yet initiated this study. .

Conclusion:

The available data preclude us to draw any conclusions.

Key Research Accomplishments

The E-cadherinW156A, when expressed in cadherin-null MiaPaCa cell line, failed to induce cell-cell adhesion compared to wild-type E-cadherin.

Reportable Outcomes:

None.

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Appendices:

None.

Supporting Data:

None.